Kidney International, Vol. 63, Supplement 85 (2003), pp. S32–S38

Chromosomal aberrations, the consequence of refractory hyperparathyroidism: Its relationship with biochemical parameters

Sandra Afonso, Íñigo Santamaría, Martín E. Guinsburg, Ana Otero Gómez, José Luis García Miranda, Rosa Jofré, Javier Menárguez, Jorge Cannata-Andía, and Juan C. Cigudosa

Cytogenetic Unit, Hospital Universitario de Canarias, Tenerife, Spain; Bone and Mineral Research Unit, Hospital Central de Asturias, Oviedo, Spain; Hospital Carlos Durand, Buenos Aires, Argentina; Nephrology Unit, Hospital Gregorio Marañón, Madrid, Spain; Pathology Service, Hospital Gregorio Marañon, Madrid, Spain; and Cytogenetic Unit, Spanish National Cancer Center (CNIO), Madrid, Spain

Chromosomal aberrations, the consequence of refractory hyperparathyroidism: Its relationship with biochemical parameters.

Background. It has been shown that refractory hyperparathyroidism (HPT) correlates biologically with a monoclonal true neoplasm, but the chromosomal changes and their relationship with biochemical variables such as high levels of phosphate, low levels of calcium (Ca), and calcitriol deficiency are still in need of a deeper analysis.

Methods. Comparative genomic hybridization was used to scan for DNA copy number changes in two groups of samples: 57 glands from refractory secondary HPT and 28 glands from refractory HPT after kidney transplantation. Biochemical HPT-related parameters from these patients were collected and analyzed.

Results. Sixty-one percent of the glands from dialysis patients and 53.6% of the glands from transplanted patients suffering severe secondary hyperparathyroidism had clonal chromosomal imbalances. Losses were far more common than gains. The most recurrent changes were losses of 1p (71%), monosomies of chromosomes 19 and 22 (45%), and losses of 20q (44%) and 16p (42%). The most frequent gains were 5q, 6q, and 13q. Biochemical parameters suggested that Ca excess is related to the development of these chromosomal aberrations, although it is not known if it is by playing a role in producing the alterations or merely as a reflection of HPT severity. Phosphate levels, despite their known effect in increasing the proliferation of the parathyroid glands, were not related to the chromosomal aberrations found in severe secondary HPT.

Conclusion. Clonal recurrent chromosomal changes are present in more than half of the glands from patients with refractory HPT, which undergo extreme biochemical levels in hyperparathyroidism effectors. These changes support the idea of the monoclonal neoplastic nature of this disorder.

Key words: genetic changes, renal failure, progression.

Hyperplasia of the parathyroid glands is a common secondary event that occurs in patients suffering chronic renal failure. As a result of this, parathyroid hormone (PTH) secretion increases, causing clinical problems such as hypercalcemia, bone disease, or nephrocalcinosis [1]. In the late stage of this disease, the hyperparathyroidism of some patients is almost autonomous and refractory, and the excessive PTH secretion no longer responds to biophysiologic influences or to aggressive medical treatment. Even after kidney transplantation, some patients do not recover and they can even develop hyperplastic growth, which affects the parathyroid glands with a similar autonomous and non-responsive behavior.

Little is known about the genomic changes that may take place in the parathyroid cells. It has been reported that the majority of surgically removed uremic parathyroid glands are monoclonal neoplasms, as assessed by X-chromosome-inactivation analysis [2–4]. Monoclonality suggests that genes involved in cell proliferation could be mutated, with the final effect being the overgrowth of the glands. At this moment there are not any likely candidates for this phenomenon [5]. Few studies, without conclusive results, have been published with the purpose of identifying chromosomal regions that may harbor putative parathyroid tumor suppressor genes [6]. In an excellent recent report of 30 uremic patients with secondary or tertiary (refractory) hyperparathyroidism, Imanishi et al [6] found, by using comparative genomic hybridization (CGH) and genome-wide allelotyping, that some chromosomal changes and allelic losses were recurrent in their study.

We have conducted a blind retrospective CGH study on two cohorts of samples from patients with secondary hyperparathyroidism (HPT) and hyperparathyroidism developed after kidney transplantation. CGH is a dou-

ble-color hybridization procedure that provides, in a single experiment, a general view of genomic monosomies or amplifications within the tumor genome. Our purpose was to detect, if any, the nature of the genetic changes involved in these two conditions and to compare the changes between both types of HPT.

The studies were complemented correlating the biochemical data obtained from these patients during their pre-dialysis, dialysis, and post-transplant (if they were transplanted) periods with the CGH results. High levels of phosphate (P), low levels of calcium (Ca), parathyroid hormone (PTH) and calcitriol deficiency are three of the main effectors in secondary HPT genesis and progression [7], high P being a likely candidate for parathyroid gland growth and monoclonality-related autonomy apparition due to irreversible structural and genetic changes [8]. The aim of this portion of the study was to establish the association between those parameters (P, Ca, and PTH) and the presence of chromosomal alterations in parathyroid glands from hemodialysis patients.

METHODS

Patients and samples

We studied 57 parathyroid glands corresponding to 22 dialysis patients with refractory secondary HPT, and 28 glands from 13 kidney-transplanted patients with refractory HPT. DNA was phenol-chloroform extracted from frozen tissue in all instances according to standard procedures.

Clinical data and statistics

Informative biochemical data was obtained from 69 glands from 29 patients who underwent parathyroidectomy. The data obtained before the parathyroidectomy were grouped according to values exceeding the normal range ($P > 5.5$ and $Ca > 10.5$); maximum values for P, Ca, and PTH were also considered. The statistical analysis (Mann-Whitney test) was performed considering the glands independently or grouping them according to the patients; they were also grouped considering those patients/glands that had received a renal transplant before the parathyroidectomy.

CGH

CGH was performed as described previously [9, 10]. Appropriated negative and positive controls were used for each experiment, and reverse labeling and fluorescence in situ hybridization (FISH) was performed in some samples to double-check our results. Standardized protocols were used for labeling and competitive hybridizations. Briefly, the probe tumor DNA was labeled by standard nick-translation using fluorescein-12-deoxyuridine-5-triphosphate. Texas Red commercially labeled normal DNA was used as reference DNA (Vysis, Inc., Downers Grove, IL, USA). Equal amounts (1 μg) of tumor and normal reference DNA were co-precipitated with 15 μg of unlabeled human Cot-1 DNA. Probe DNA was resuspended in 7 μL of hybridization mixture (50 formamide, 2X SSC, 10 dextran sulfate) and hybridized to normal human metaphase chromosomes slides commercially available (Vysis, Inc.). Hybridization was performed at 37°C for 48 hours. The slides were washed three times (5 minutes each) at 45°C in 2X SSC, followed by three washes (5 minutes each) at 45°C in 0,1X SSC, and one wash (10 minutes) at 45°C in 1X PBD. Slides were counterstained with 4,6-diamino-2-phenylindole (DAPI) for the identification of chromosomes.

Digital image analysis

The green and red fluorescence intensities of the hybridization signals and the 4,6-diamino-2-phenylindole staining patterns were recorded with a cooled charge-coupled device camera attached to a Nikon Eclipse 400 microscope (Nikon, Natick, MA, USA). Fluorescence ratio profiles for each chromosome were calculated by using the Quantitative Image Processing System (Vysis, Inc.). For each hybridization, the data from 10 to 14 representations of each chromosome were combined to yield the mean and 95% confidence interval for that ratio, plotted next to the ideogram for that chromosome. Gains or losses of chromosomes or chromosomal regions were detected on the basis of ratio profile deviations from the green/red balance value of 1.0. The upper and lower threshold limits for defining chromosomal gains and losses were set at 1.20 and 0.80, respectively. These threshold values were determined in CGH experiments using two differentially labeled, normal genomic DNA samples. In these negative control experiments, the mean green/red ratio was well within the range of 1.20 to 0.80 for the entire length of all chromosomes, providing robust, highly stringent criteria for the determination of gains and losses in tumor samples. Metaphase spreads with uniform high-intensity fluorescence in both green and red colors on both homologous chromosomes, and with no background spots, were selected for evaluations. The centromeric and heterochromatic regions and p arm of acrocentric chromosomes and telomeric regions were not included in the interpretation of gains and losses.

RESULTS

Chromosomal changes detected by CGH

Fifty-seven parathyroid glands from 22 dialysis patients with secondary HPT were analyzed by CGH. Thirty-five glands (61%) demonstrated clonal chromosomal imbalances that included both gains and losses of whole or partial chromosomes. Losses were far more common than gains. The abnormal cases presented a mean of 3.7 losses (range, 0–12) and of 1.2 gains (range, 0–10). The

Table 1. Frequencies of the most recurrent chromosomal changes in refractory secondary hyperparathyroidism and after kidney transplantation hyperplastic parathyroid glands

Frequencies of chrosomosomal losses %			Frequencies of chrosomosomal gains %		
Chromosome region	Secondary HPT	After transplantation HPT	Chromosome region	Secondary HPT	After transplantation HPT
1p33–pter	71.4	73.3	4q22–q27	11.5	13.5
9q	22.9	6.6	5q11–q22	22.9	6.6
12q22–qter	17.1	26.6	6q12–q21	25.7	13.3
16p	42.9	20	12q13–q21	11.4	6.6
17p/q	31.4	20	13q21	26	0
19	45.7	26.6			
20q	44.4	33.3			
22	45.7	26.6			

HPT is hyperparathyroidism.

frequencies of each change, related to the number of abnormal glands, are shown in Table 1. The chromosomal region 1p35-pter was the most common loss (71% of abnormal cases), followed by monosomies of chromosomes 19 and 22 (45%), and losses of 20q (44%) and 16p (42%). As for gains, the most frequent were gains of 5q, 6q, and 13q. The exact cytogenetic locations of the chromosomal changes are shown in Figure 1. Among the abnormal glands, only 6 samples showed single changes. The remaining glands showed two or more changes. The single changes were: loss of 1p34-pter (in 3 cases), gain of complete chromosome 5 (2 cases), and loss of 3q26-qter (1 case).

Twenty-eight parathyroid glands from 13 kidney-transplanted patients with refractory HPT were analyzed by CGH. Fifteen glands (53.6%) displayed genomic changes, including gains and losses. The abnormal cases presented a mean of 3.2 losses (range, 0–9) and a mean of 0.5 gains (range, 0–2). The proportion of changes is also shown in Table 1. The specific chromosome regions that were altered are shown in Figure 2. The chromosomal region 1p33-pter was the most common loss (73% of abnormal cases) followed by losses of 20q (33%), monosomies 19 and 22, and loss of 12q (26% of cases). As for gains, the most frequent were gains of 4q and 6q (13%). Among the abnormal glands, only 3 samples showed single changes, the remaining showing two or more changes. In those samples, the single genomic changes were: loss of 1p33-pter (in 1 cases), gain of chromosome 5q (1 case), and loss of 8q22-qter (1 case).

Comparison of the frequencies of both set of patients (Table 1) revealed that the group with secondary HPT showed a slightly higher number of samples with chromosomal changes, but the difference was not statistically significant ($P > 0.5$). The type and relative frequency of the chromosomal aberrations were similar in both sets of samples. It is noteworthy that two abnormalities have also been found as a unique change in both sets of samples: loss of 1p and gain of 5q.

Clinical associations

Eighty percent of the patients with informative biochemical data available showed alterations in at least one of the extracted glands. As can be seen in Figure 3, maximum values of calcium are significantly related to the presence of chromosomal aberrations, either when counting glands or patients. The data have been considered in both ways because, although the glands from the same patient are exposed to the same levels of P and Ca, they could behave independently in their response. No significant differences were found regarding PTH levels or time on dialysis, perhaps due to the great variability in the control and treatment of these patients.

When considering the glands as independent values, maximum levels of P and also mean levels above the normal range of P (markers of prolonged hyperphosphatemia) have a significant inverse relationship with genetic alterations detected by CGH. Thus, contrary to what was expected, maximum P and excess of P did not correlate with the presence of chromosomal aberrations.

The data was also subgrouped according to the patients/glands that had received a kidney transplant before the parathyroidectomy. The same tendency was observed in both batches of data, although only maximum Ca and P were significantly different (the former in both glands and patients, not transplanted and transplanted, respectively, and the latter in transplanted glands. Data not shown.)

DISCUSSION

Refractory hyperparathyroidism is a common event in long-term chronic renal failure. However, our knowledge about the biological process that occurs within the parathyroid glands of these patients is quite limited. The first question about the nature of these disorders was to assess if it was a polyclonal expansion or a monoclonal neoplasm. It has been demonstrated by X-chromosome-inactivation analysis [2–4, 11], that a large majority of the patients with refractory hyperparathyroidism exhibit

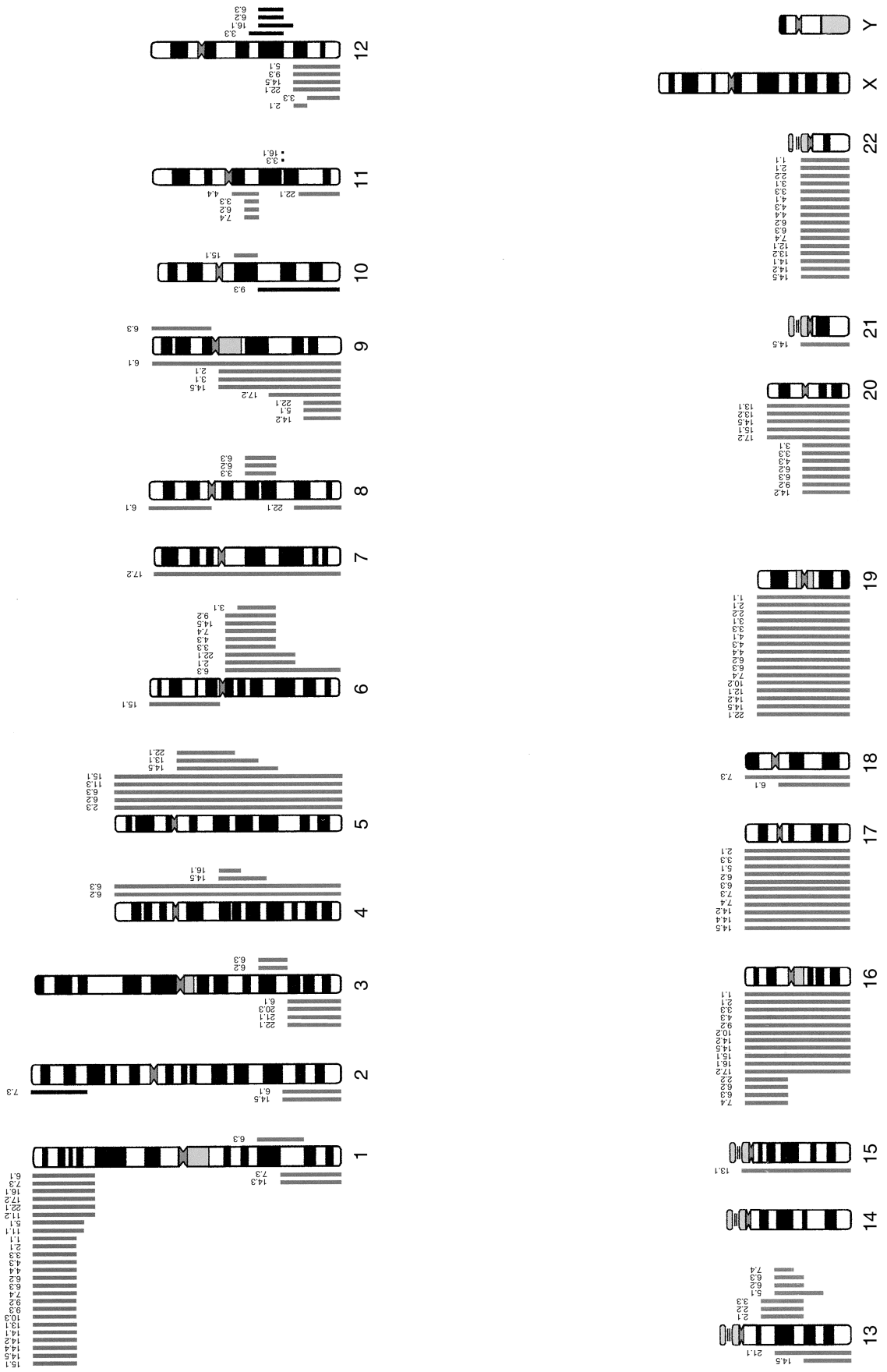

Fig. 1. DNA copy number changes in 57 parathyroid glands from 22 patients with refractory secondary hyperparathyroidism. Each vertical bar on the left side of each chromosome represents a loss in a particular gland and indicates the size and localization of the change. Each bar on the right side of the chromosome represents a gain of DNA of that particular chromosome or chromosomal region.

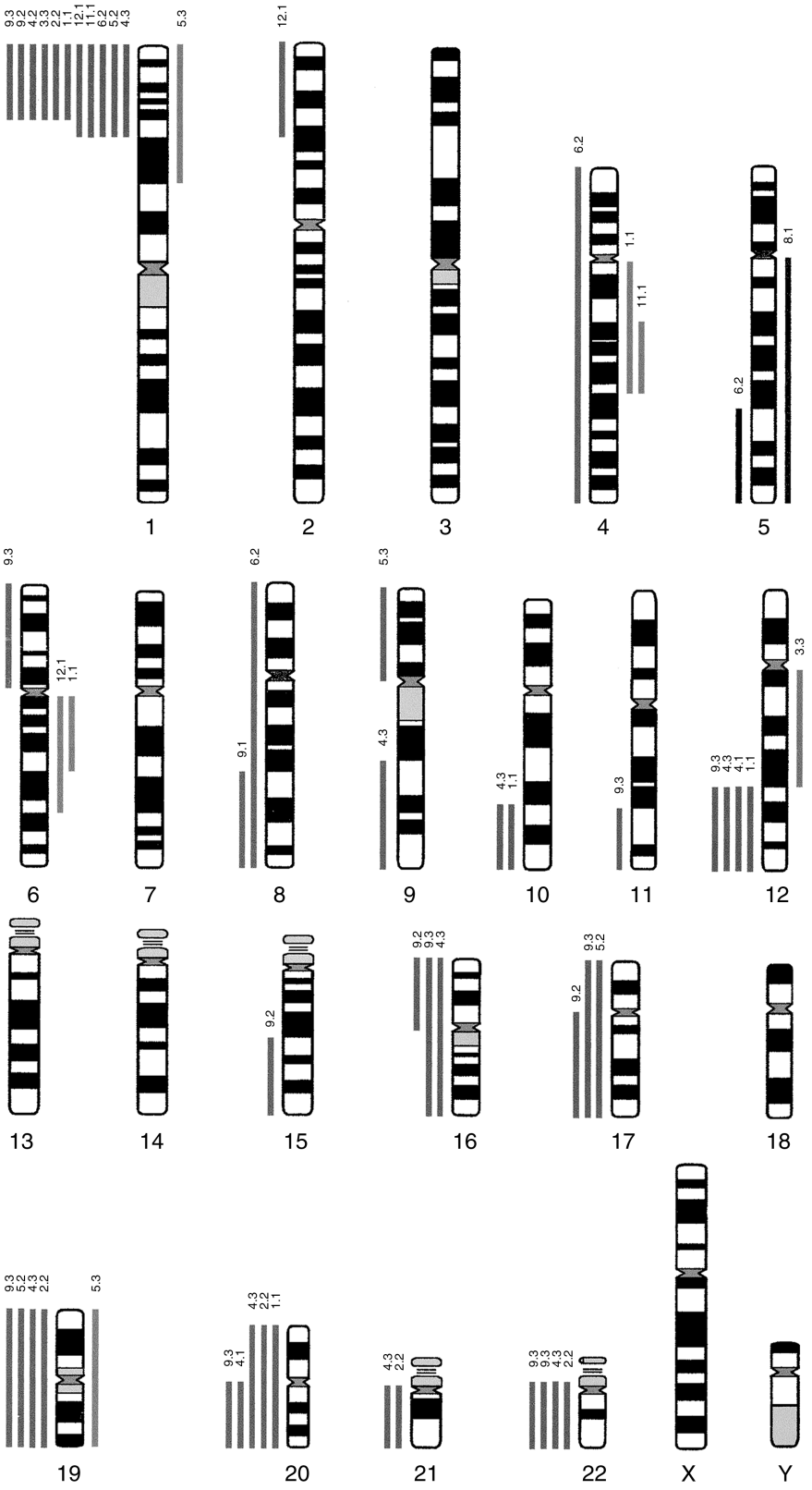

Fig. 2. DNA copy number changes in 28 parathyroid glands from 13 patients with refractory hyperparathyroidism after kidney transplantation. Each vertical bar on the left side of each chromosome represents a loss in a particular gland and indicates the size and localization of the change. Each bar on the right side of the chromosome represents a gain of DNA of that particular chromosome or chromosomal region.

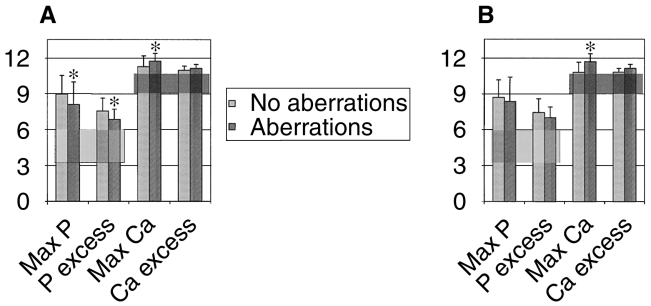

Fig. 3. Relationship between the levels of P and Ca and the presence or absence of chromosomal aberrations. (*A*) Glands considered independently and (*B*) data grouped according to the patients. Light gray square = normal range of values for P. Dark gray square = normal range of values for Ca. $*P < 0.05$

a true neoplastic disorder. According to these observations, a recent report [6] has shown that in a series of 46 parathyroid samples, 24% of them harbored one or more chromosomal changes detected by CGH. These authors also mentioned that the true number of monoclonal tumors is likely to be much higher. In agreement with this hypothesis, in our study of 85 samples, the proportion of cases with chromosomal changes was higher (>50%), probably reflecting the severe conditions of the patients with enriched monoclonal neoplasms [6]. Technical overestimation of the chromosomal changes was ruled out by two means. First, we performed reverse CGH by labeling the reference and test DNA with the opposite colors, in order to double-check the results. Second, we performed fluorescence in situ hybridization (FISH), with probes from chromosomes 19 and 22 on positive samples. The results of these experiments were always confirmatory.

The nature of the chromosomal changes in our series showed some specific features when it was compared with previous reports. In the reported series of uremic patients [6], the most common changes were gains of chromosomes 7 and 12, changes that did not appear as relevant in our series. We found some coincidences, such as the gain of the 6q region. The differences could be explained by the number of samples and by the clinical condition of the selection of patients. The results of a CGH study performed on parathyroid adenomas [12] showed some similarities and differences with the changes that we have found in our series. Loss of 1p is one of the most relevant findings in adenomas and it is also the most recurrent chromosomal aberration in our group of samples. In a similar way, losses of 9q and 11q are also found in adenomas and our samples. The main difference between the reported series of adenomas [12] and our series is that we have found a higher number of monosomies/losses involving chromosomes 1, 16, 17, 19, 20, and 22. The observed gains of 5q and 6q, which have not been reported previously, could be a hallmark of the secondary HPT.

It is important to note that some chromosomal aberrations were seen as unique changes in the samples. Loss of 1p and gain of 5q were the most relevant. The fact that these anomalies are the only change confers a putative role in the pathogenesis of the disease. More molecular work needs to be done to clarify the exact role of the genes that are contained in those particular chromosome regions.

The comparative CGH-biochemical portion of the study suggests that Ca excess could be related to the development of chromosomal aberrations, although it is not known if it plays a role in producing chromosomal alterations, or if it is merely a reflection of secondary HPT severity. High phosphate levels, a well-known factor able to increase the proliferation of the parathyroid glands, was not related to the rise of chromosomal aberrations found in severe secondary HPT in this study. This could point out that the merging of chromosomal aberrations in secondary HPT is not due to the mere proliferation increase by P levels, but to improper proliferation mechanisms triggered by still unknown factors during the worsening of secondary hyperparathyroidism. Furthermore, the data shown here suggest that severity of the hyperplasia maybe not be equal to genetic damage in parathyroid glands. Further studies with a larger number of glands are needed to complete and validate these novel findings.

ACKNOWLEDGMENTS

Dr. Iñigo Santamaria is a recipient of a Research Contract from Fondo Investigaciones Sanitarias (FIS 00/3161).

Reprint requests to Dr. Juan C. Cigudosa, Cytogenetics Unit, CNIO (Spanish National Cancer Center) C/Melchor Fernandez Almagro, 3, 28029 Madrid, Spain.
E-mail: jccigudosa@cnio.es

REFERENCES

1. Indridason OS, Quarles LD: Tertiary hyperparathyroidism and refractory secondary hyperparathyroidism, in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, 4th edition, edited by Favus MJ, Philadelphia, Lippincott Williams & Wilkins, 1999, pp 198–202
2. Arnold A, Brown MF, Ureña MF, *et al*: Monoclonality of parathyroid tumors in chronic renal failure and in primary parathyroid hyperplasia. *J Clin Invest* 95:2047–2053, 1995
3. Tominaga Y, Kohara S: Namii Y, *et al*: Clonal analysis of nodular parathyroid hyperplasia in renal hyperparathyroidism. *World J Surg* 20:744–750, 1996
4. Chdek J, Ritz E, Kovacs G: Genetic abnormalities in parathyroid nodules of uremic patients. *Clin Cancer Res* 4:211–214, 1998
5. Brown SB, Brierley TT, Palanisamy N, *et al*: Vitamin D receptor as a candidate tumor-suppressor gene in severe hyperparathyroidism of uremia. *J Clin Endocrinol Metab* 85:868–872, 2000
6. Imanishi Y, Takahara H, Palanisamy N, *et al*: Clonal chromosomal defects in the molecular pathogenesis of refractory hyperparathyroidism of uremia. *J Am Soc Nephrol* 13:1490–1498, 2002
7. Tominaga Y, Sato K, Tanaka Y, *et al*: Histopathology and patho-

physiology of secondary hyperparathyroidism due to chronic renal failure. *Clin Nephrol* 44(Suppl 1):S42–47, 1995
8. Slatopolsky E, Dusso A, Brown AJ: The role of phosphorus in the development of secondary hyperparathyroidism and parathyroid cell proliferation in chronic renal failure. *Am J Med Sci* 317:370–376, 1999
9. Houldsworth J, Chaganti RSK: Comparative genomic hybridization: An overview. *Am J Pathol* 145:1253–1260, 1994
10. Cigudosa JC, Rao PH, Calasanz MJ, *et al*: Characterization of nonrandom chromosomal gains and losses in multiple myeloma by comparative genomic hybridization. *Blood* 91:3007–3010, 1998
11. Shan L, Nakamura Y, Murakami M, *et al*: Clonal emergence in uremic parathyroid hyperplasia is not related to MEN1 gene abnormality. *Jpn J Cancer Res* 90:965–969, 1999
12. Palanisamy N, Imanishi Y, Rao PH, *et al*: Novel chromosomal abnormalities identified by comparative genomic hybridisation in parathyroid adenomas. *J Clin Endocrinol Metab* 83:1766–1770, 1998